Application No. 10/758,237

REMARKS

This Amendment is filed in response to the Office Action dated February 13, 2008 (the Office Action).

Claims 45-55, 58-61, 63-67, and 69-84 are cancelled herein for future divisional prosecution.

Claims 5-8, 36, 38-40, and 98-101 have been withdrawn by the Examiner as being directed to a species not under present examination. Rejoinder is requested upon allowance of a generic or linking claim.

Claims 1, 19-23, 25-28, 31-35, 37, 41-43, 85-87, 90-94, and 102 are under examination.

Claims 23 and 26 are amended as described below.

Regarding the Office Action's objections to the Sequence Data, Applicant encloses a statement referring to the February 13 and May 21 submissions with the required statements.

35 U.S.C. §112 rejections of claims 23 and 26

Claim 23 stands rejected as being indefinite under 35 U.S.C. § 112¶2. This claim has been amended to remove the misplaced Markush language. Withdrawal of this rejection is requested.

Claim 26, at page 12 of the Office Action, is rejected under 35 U.S.C. 112 ¶2 as being indefinite. This claim was amended to require an independent promoter for the suicide sequence nucleic acid relative to promoters for the exogenous nucleic acid.

35 U.S.C. §103(a) rejections of claims 1, 19-21, 23, 27, 28, 31-35, 41-43, 85-87, 90-94, and 102

Claims 1, 19-21, 23, 27, 28, 31-35, 41-43, 85-87, 90-94, and 102 stand rejected under 35 U.S.C. §103(a) in light of WO 98/40510 (Hackett et al.) in view of U.S. Pat. No. 5,610,033 (Chung et al.). Also, claims 1, 25, and 26 stand rejected under 35 U.S.C. §103(a) in light of WO 98/40510 (Hackett et al.) in view of U.S. Pat. No. 5,610,033 (Chung et al.) and in further view of Pope et al. (1997) Eur. J. Cancer 33:1005-1016.

The Office Action's obviousness rejections are traversed, in general, on the grounds that (1) the rationale provided for the *prima facie* case is, with all due respect, unfounded, and because (2) the testimony of experts shows that what is claimed is not obvious. These points are detailed below.

<u>Applicant traverses the Examiner's prima facie case of obviousness of the grounds that it has an incorrect rationale; instead, the prior art teaches away from the proposed combination of references.</u>

The Office Action states that there is "no basis for concluding that the mechanism of Sleeping Beauty transposase is incompatible with the insulator element of Chung et al.", or that if there were "some interference" that "the level of skill in the art is very high" such that "routine experimentation" would be adequate to overcome the same, e.g., "by determining the optimum positioning of the elements relative to one another". The Office Action points to the record for the Patent Office's previous rejections and Applicant's arguments. Accordingly, only the points raised in the Office Action will be addressed herein. It is submitted that the prior art teaches away from the combination of transposon and the insulators, as explained below.

By way of background, Sleeping Beauty is a transposase that binds to Class II DNA transposons. The transposons are DNA sequences. The binding by Sleeping Beauty transposase requires the transposons (DNA sequences) to form a loop by binding to each end of the transposon (see previous amendment for detailed citations). Sleeping Beauty is an enzyme that cuts-and-pastes the transposon into the host DNA. The claimed transposon has an insulator. Insulators make DNA around them loop, although the shape of that loop is unclear.

The figure on page 9 of the Office Action shows looped <u>transposons</u> and hypothesizes that looping caused by insulator elements would facilitate transposon activity by bringing the ITRs [inverted terminal repeats] on the transposons into closer proximity. But bringing the ITRs into closer proximity is the function of the Sleeping Beauty transposase. An insulator process that competes with the function of the Sleeping Beauty transposase would be expected by the ordinary artisan to <u>reduce</u> the transposase's activity, as by competitive inhibition. Thus the prior art leads the artisan away from using insulators. As further evidence, Schleif and his students

[e.g., Lee and Schleif (1989) "In vivo DNA loops in araCBAD: Size limits and helical repeat.", Proceedings of the National Academy of Sciences USA 86: 476-480, attached], looping can be subtle when conducted by proteins, as in this case. Cui et al. (2002) followed the teachings of Schleif et al. to make deletions that take out integral turns of the DNA helix (Fig. 3, constructs Δ60L, Δ50M and Δ50R) but did NOT achieve the expected outcomes! Hence, Cui et al. (2002) teaches the ordinary artisan to avoid using systems that rely of particular insulator loop behaviors. Merely moving the insulator sequences does not directly address the looping interference problem.

<u>Applicant traverses the Examiner's rejection on the grounds that experts have testified to the innovative character of the invention.</u>

Experts examined the claimed transposon-insulator combination close to the time of filing and agreed that this combination is highly innovative. The praise and testimony of objective and independent experts provided herein is strong and direct evidence that artisans would not combine the references as outlined in the Office Action. This testimony is also objective secondary evidence that suited to overcoming a prima facie case of obviousness such that the claims can be allowed even if the Patent Office were to maintain its present position despite the reasons presented herein.

Objective third-party experts retained by the National Institutes of Health (NIH) reviewed the invention as part of the Developmental Biology Subcommittee, see attached Review. Their review was completed in September 2003. This review was performed as part of a grant-review process, with Dr. Hackett (Applicant) being a grant applicant. The NIH assembles panels of experts in committees to rank research proposals. At page 12, Project IV is identified as the Sleeping Beauty Transposon for Gene Therapy. At page 13, ¶3 of the Review, Specific Aim 1 is noted as describing the claimed insulator-transposon for Sleeping Beauty transposase that is presently claimed, see also page 14, third paragraph from the bottom.

A first independent expert states, at page 14 ¶2, that "this is a highly innovative project. This is one of the first and continues to be one of the only laboratories working on transposable

elements as a method for gene transfer. The investigators have pioneered many aspects of this field". The reviewer, at page 14 ¶ 3 notes that Dr. Hackett is "uniquely qualified" for the project.

A second independent expert also reviews the project in detail and states, at page 15, ¶2, that "the proposed experimental approach is highly innovative".

Both independent experts provide objective testimony from an independent U.S. government agency that the claimed invention is highly innovative. This testimony is given more than about five years after the publication of Hackett et al. or Chung et al. Even after a significant amount of time had passed in these arts, these objective experts found the claimed approach not only to be "innovative" but "highly" innovative at the time of filing.

35 U.S.C. §103(a) rejection of claim 22

Claim 22 has been rejected under 35 U.S.C. §103(a) as being unpatentable over Hackett et al. (1998) WO 98/40510 in view of Chung et al. cited above and in further view of Wooddell et al. Claim 22 is patentable for the reasons described above and Woodell et al. does not make up for the deficiencies of Chung et al. and Hackett et al.

Request for allowance

Withdrawal of the rejections, rejoinder of the withdrawn claims, allowance of all of the claims is requested.

The undersigned would welcome a telephone call if the Examiner believes it would be useful to advance prosecution.

Respectfully submitted,

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Biochemistry

In vivo DNA loops in *araCBAD*: Size limits and helical repeat

(supercoiling/twist/periodicity/linking number deficit/looping energetics)

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Communicated by Alexander Rich, October 14, 1988

ABSTRACT **Formation of a DNA loop by AraC proteins bound at the *araI* and *araO*$_2$ sites, whose center-to-center distance is 211 base pairs, is necessary for repression of the *araBAD* promoter, P_{BAD}, of *Escherichia coli*. To determine the upper and lower size limits of the loop, we constructed P_{BAD}–reporter gene fusion plasmids with various spacings between *araI* and *araO*$_2$ and measured their levels of expression. Spacings larger than about 500 base pairs resulted in elimination of detectable repression. No lower limit to spacing was found, suggesting that AraC protein itself possesses significant flexibility and its bending substantially aids formation of small loops. As the spacing between *araI* and *araO*$_2$ varied, the activity of P_{BAD} oscillated with an 11.1-base-pair periodicity, implying that the *in vivo* helical repeat of this DNA is 11.1 base pairs per turn.**

Regulation of the repressed basal level as well as the induced level of expression of the arabinose operon in *Escherichia coli* involves DNA looping mediated by the AraC protein. AraC proteins bound to two distinct DNA sites and bound to each other to form a DNA loop (1–4). DNA looping in the *ara* system requires that the two binding sites involved in forming the repression loop, *araI* and *araO*$_2$ (Fig. 1), be on the correct face of the DNA double helix (1, 2). Although the wild-type spacing of 211 base pairs (bp) between *araI* and *araO*$_2$ gives rise to full repression of P_{BAD}, insertion of 5 bp between the two sites rotates one site halfway around the DNA double helix with respect to the other site and greatly interferes with repression. We interpret this finding as resulting from the torsional stiffness of DNA. Forcing the two misaligned sites to the same side of the DNA, so that looping could occur, would cost about 2.1 kcal/mol (1 cal = 4.184 J) (5) and could interfere with repression if the AraC protein does not have sufficient lateral flexibility to overcome misalignment of its binding sites.

Measurements on bare linear DNA made *in vitro* have yielded values close to 10.5 bp per turn for average DNA (6–8). The helical repeat of DNA *in vivo* apparently has not been measured, but we might expect it to possess a slightly different value because on average such DNA possesses a linking number deficit. This deficit generates supercoiling (9) and, in principle, could also partially untwist the DNA. Indeed, an untwisting caused by a linking number deficit has been observed *in vitro* for supercoiled DNA (10, 11). Another reason for deviation from the canonical value of 10.5 bp per turn might be local supercoiling generated by transcription of adjacent genes (12). As the spacing between *araI* and *araO*$_2$ is progressively increased or decreased, repression of P_{BAD} should oscillate with a period about equal to the helical repeat of the DNA. Therefore, we have utilized looping in the *ara* system to measure the helical repeat *in vivo* of the DNA of the *ara* operon regulatory region.

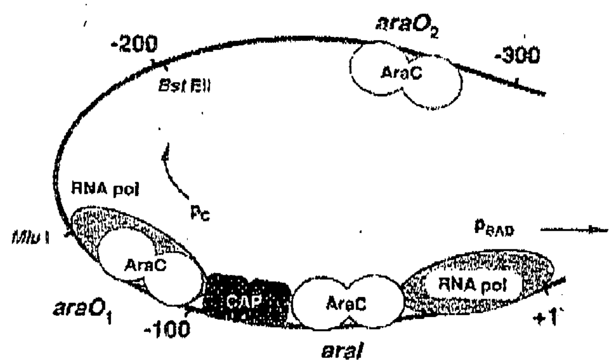

FIG. 1. Protein-binding sites in the L-arabinose *araCBAD* regulatory region. Numbering of base pairs is relative to the P_{BAD} transcription start at position +1. Stippled area, RNA polymerase; open area, AraC protein; solid area, cAMP CAP.

Both upper and lower limits to the DNA loop size ought to exist. As the length of the DNA separating *araI* and *araO*$_2$ increases to large values, the sites should have increasing difficulty finding one another and looping should decrease. At the other extreme, when the length of DNA separating the sites decreases, the stiffness of the DNA should hold the sites (and the proteins bound to the sites) apart and hinder loop formation. Maximal loop formation ought to occur at some intermediate loop size, much like the maximum that is seen in DNA ring closure or cyclization as a function of DNA length (5, 13, 14).

Several factors other than loop size may also affect DNA looping. Supercoiling may compact DNA and assist loop formation. Proteins other than AraC—for example, HU, IHF, FIS, or catabolite-activating protein (CAP) (15–18)—may bind to and bend the DNA or change its helical repeat. Finally, flexibility of the AraC protein itself could assist formation of small loops.

Here we report experiments showing that the helical repeat of the DNA between *araI* and *araO*$_2$ is 11.1 bp per turn, that the upper size limit of the *ara* loop is about 500 bp, and that there is no lower size limit to this loop.

MATERIALS AND METHODS

Media, Strains, and General Methods. Media and general methods have been described (19, 20). Plasmid pTD3 contains 440 bp of the *araCBAD* regulatory region on a *Hin*dIII–*Eco*RI fragment and has P_{BAD} driving *galK* of the pKO1 vector (1). Its derivative, pLH2 (4), contains the M13 replication origin at the end of the *galK* structural gene. Plasmid pDL3 was made by replacing the *galK* structural region of pLH2 with the *lacZ* structural region of pPN10 (ref. 21; kindly donated by P. Norton, Tufts University School of Medicine) while maintaining the *galK* leader region. The AraC-overproducing plasmid,

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Abbreviation: CAP, catabolite-activating protein.

pDL5, combines the gene coding for AraC under the control of the *lacUV5* promoter (22) and the vector part of pGA44 (23). It has the p15A replication origin that is compatible with the colEI replication origin of pDL3, the chloramphenicol drug-resistance marker, and it synthesizes AraC protein at greater than 100 times the rate of the wild-type chromosomal copy of *araC*. Strains used were the $AraC^-$ strain SH321, its isogenic partner $AraC^+$ SH322, F^+ SH322 (called DL100), and $AraC^+$ cya^- SH326 (24). Strain DL110 contains the mutation *gyrB225* introduced into SH322 by P1 transduction from AE400 (F^- *trpB gyrB225 tna*::Tn*10 ilvO*; a gift of A. Wright, Tufts University School of Medicine), a derivative of AE199 (25). The linking number deficit in plasmids extracted from this strain was about −0.03, as measured by band counting of topoisomers on chloroquine-containing agarose gels (26). Plasmids from the strain lacking the *gyr* mutation had an average linking number deficit of −0.06.

Construction of Insertion Plasmids. We use the distance between the apparent middle of the inverted sequence symmetrics of the *araI* (position −59) and $araO_2$ (position −270) sites when discussing the distance between them. Plasmid pTD3 was cut with the restriction enzyme *Bst*EII, treated with the Klenow fragment of DNA polymerase I and alkaline phosphatase, and ligated with *Hae* III fragments of *E. coli* chromosomal DNA smaller than 1000 bp long. The strain SH322 was used for transformation and scoring of insertion candidates on MacConkey galactose-indicating plates. The size of each insertion was determined by electrophoresis of the *Hin*dIII–*Eco*RI segment from the plasmids and comparison to sequenced size markers. Several candidates were chosen for further investigation by sequence analysis, isolation of repression-defective mutations, and investigation of helical twist dependency by addition of 4- or 8-bp insertions.

Small-sized insertions (+13, +15, +17, +19, +21, +23, +25, +29, +31, +33, and +35 bp) were made by inserting oligonucleotides of various sizes into the *Bst*EII site of pLH2 and subsequent insertion of 4 nucleotides in restriction sites contained on the oligonucleotides. Four plasmids having spacing changes of −16, −8, +5, and +11 bp from the wild-type spacing (211 bp) were described in Dunn *et al.* (1). The promoter fragments of these spacing mutants were recloned into the vector part of pDL3 to allow P_{BAD} promoter activities to be measured by β-galactosidase assay.

Construction of Deletion Plasmids. Plasmid pLH2 was cut with *Bst*EII, partially digested with slow BAL-31 exonuclease (27), ligated, and transformed into strain SH322. Approximately 400 candidates were collected, and the sizes of their deletions were determined by comparing their *Hin*dIII–*Eco*RI fragments with known size markers. The candidates having deleted less than 200 bp, which might retain intact *araI* and $araO_2$ sites, were chosen for further investigation by sequence analysis, isolation of repression-defective mutations, investigation of helical twist dependency by addition of 4-bp insertions, and *in vivo* footprinting.

Some of the important spacing plasmids discussed later delete the following regions: for a 32-bp spacing, positions −81 to −259; for a 33-bp spacing, positions −82 to −259; for a 34-bp spacing, positions −84 to −260; for a 38-bp spacing, positions −77 to −249; for a 44-bp spacing, positions −82 to −248; for a 49-bp spacing, positions −88 to −249; for a 111-bp spacing, positions −158 to −257; and for a 146-bp spacing, positions −164 to −228. The numbering of positions is relative to the P_{BAD} transcription start at position +1.

Measurement of Promoter Activities. Plasmid constructs possessing an intact CAP binding site were transformed into DL100 cells, whereas plasmid constructs having a defective CAP binding site were transformed into SH326 cells harboring pDL5. The new strains were grown in M10 medium (19) for at least five generations to a density of 2×10^8 cells per ml. The β-galactosidase assays and units described by Miller (28) and the galactokinase assays (24) were used depending on the structural gene of the test plasmids. The typical standard deviations of the β-galactosidase and galactokinase assays were 8% and 11%, respectively.

Other Methods. Detailed methods for the isolation and analysis of repression-defective mutants and *in vivo* footprinting were described (3). Fourier transformation (29) of the repression and induction properties as a function of the *araI*–$araO_2$ spacing was performed with a spread-sheet program. For this operation the induced enzyme levels in the spacing plasmids without the CAP binding site (spacing, 32–86 bp) were multiplied by 0.1 before transformation, and the value of the enzyme levels for spacings that were not constructed was taken to be zero. The amplitude (A) as a function of the periodicity (p) was evaluated for p between 9 and 13 bp, where s is the spacing between *araI* and $araO_2$ and $E(s)$ is the enzyme level measured for the spacing.

$$A(p) = \sqrt{\left[\sum_s E(s)\sin\left(\frac{2\pi s}{p}\right)\right]^2 + \left[\sum_s E(s)\cos\left(\frac{2\pi s}{p}\right)\right]^2}.$$

The approximate relative energies of loop formation were determined by an analog technique. We measured the work required to bend elastically a series of flexible steel strips with various lengths, obtained from a surveyor's tape, from straight to a conformation in which the ends were a scaled distance of 150 Å apart, corresponding to twice the estimated diameter of AraC protein. During this process the ends were not angularly constrained. This freedom corresponds to flexibility in the protein.

RESULTS

Loop Size Upper Limits. We concentrated on inserting DNA fragments smaller than 2000 bp because separating the *araI* and $araO_2$ sites by about 2000 bp yielded only repression-negative derivatives. About 1000 candidates were generated by inserting *Hae* III fragments of *E. coli* chromosomal DNA smaller than 1000 bp long into the *Bst*EII site of pTD3. One hundred and three candidates were repression-positive or partially positive and were examined further. We also chose 41 repression-negative colonies for additional characterization. Fig. 2*a* shows the relationship between the approximate size of the insertion and the degree of repression as shown on indicating plates in which repression-negative colonies were red and repression-positive colonies were white.

In any particular size class of insertions we might expect some to leave *araI* and $araO_2$ misoriented with respect to the helical face for repression and others to leave these sites correctly oriented. Hence the most significant sizes with respect to repression or lack thereof in any size region of the graph are those that repress best. Apparently, as the loop size increases above 300 bp, looping or repression becomes increasingly difficult and is lost by about 500 bp. Seven of the 62 different-sized plasmids containing insertions of more than 50 bp were more fully characterized by DNA sequencing and quantitative measurement of repression. Data derived from these plasmids (Fig. 2*b*) agree with their more qualitative characterization.

The plasmid containing the 299-bp spacing repressed P_{BAD} as well as a plasmid containing no insertion, but the insertion of an additional 4 bp at the *Mlu* I site in this plasmid significantly interfered with repression (Fig. 2*b*). Plasmids with a 346-bp separation and a 401-bp separation between *araI* and $araO_2$ poorly repressed and their repression was not significantly improved by inserting an additional 4 or 8 bp between *araI* and $araO_2$. This indicates that the loop size itself was the reason for the poor repression. The promoter activity on the plasmid with 616 bp between *araI* and $araO_2$

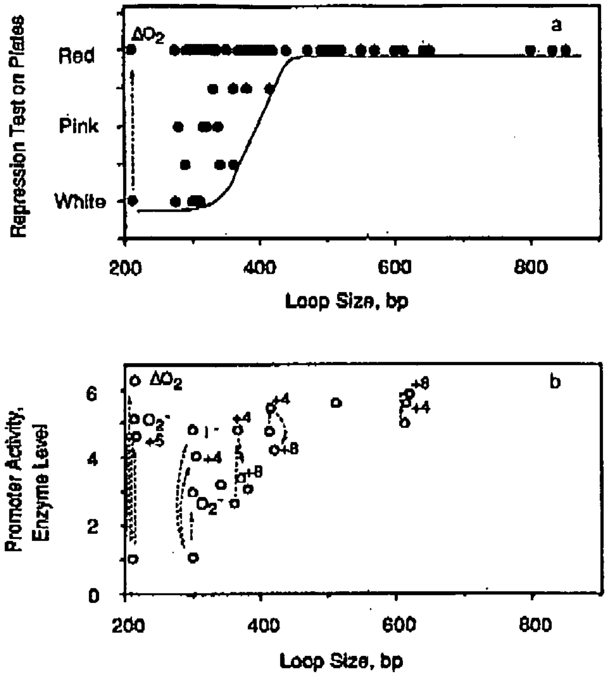

FIG. 2. Repression as a function of loop size. (*a*) Qualitative test of 62 candidates containing insertions between *araO*$_2$ and *araI*. The plasmids in strain DL100 were streaked on MacConkey galactose-indicating plates and the color of the colonies was recorded after 14 hr of growth. The solid line shows the best repression abilities in each size range. (*b*) Basal level *araBAD* promoter activities in strain DL100 by direct galactokinase assay of seven insertion plasmids and their derivatives that were chosen from the same 62 candidates shown in *a*. Broken lines indicate that subsequent basal level after addition of 4 or 8 bp at the *Mlu* I restriction site, isolation of repression-negative mutants after hydroxylamine mutagenesis of the *araBAD* promoter region (O_2^- and I^-), or deletion of the *araO*$_2$ site (ΔO_2).

remained repression-negative even after insertion of an additional 4 or 8 bp. These results show that loops in the *ara* system larger than about 500 bp cannot easily be formed with wild-type AraC protein.

Mutations isolated solely on the basis that they reduced repression in the plasmid with the 299-bp loop were located in the *araI* site (G → A at position −43) and the *araO*$_2$ site (C → T at position −271) or in the RNA polymerase binding site, just as was found by Martin *et al.* (3). This indicates that the *araI* and *araO*$_2$ sites, and not extraneous sequences on the plasmid, were required for repression in this plasmid.

Loop-Size Lower Limits. Deletions were generated to move *araO*$_2$ and *araI* closer together, and the candidates retaining intact *araO*$_2$ and CAP binding sites were further characterized.

A deletion of 100 bp, leaving a loop of 111 bp, showed normal repression. It was a surprise that loops as small as this should form, since significant work is required to bend 100 bp of DNA into a circle. That is, the persistence length of DNA in physiological buffers is about 140 bp (14). Therefore, we tested whether the looping was between *araI* and *araO*$_2$ by mutational analysis, as was done for the 299-bp spacing plasmid, and found that the *araI* and *araO*$_2$ sites in a plasmid containing the 146-bp spacing were involved in a small repression loop.

The smallest possible spacing between *araI* and *araO*$_2$ that will not damage either site or the intervening CAP site is 70 bp. Deleting or damaging the CAP site substantially lowered the repressed and induced levels of P_{BAD} (1, 30), and this would interfere with interpretation of data. In the absence of a functional CAP binding site, however, looping between *araI* and *araO*$_2$ can be detected by its dramatic reduction of the arabinose-induced levels of P_{BAD} activity when somewhat elevated levels of AraC protein are provided (24).

To examine the ability to form small loops, we constructed another AraC-overproducing plasmid, pDL5, which is compatible with the spacing mutant plasmids so that both the hypersynthesis plasmid and the P_{BAD}–*lacZ* plasmid could be stably maintained in cells. The inducibility of P_{BAD} in this system as a function of the *araI*–*araO*$_2$ spacing was compared to the repression abilities in the CAP$^+$ plasmids with the same spacings. As shown in Fig. 3*c*, both inducibility in the CAP$^-$ system and repression in the CAP$^+$ system show identical loop size dependencies for loops around 140 bp and loops around 230 bp. That is, the maxima and minima of the basal levels occur at the same loop sizes as the maxima and minima of the induced levels. Therefore, we extended our examination of loop formation to spacings of less than 70 bp between *araI* and *araO*$_2$ when part or all of the CAP site was deleted.

The inducibility of P_{BAD} in the plasmids with the CAP site deleted oscillated as the spacing between *araI* and *araO*$_2$ varied as shown in Fig. 3*c*. This oscillation retained the same periodicity of about 11 bp and the same phase as the oscillations in the basal level observed with the plasmids

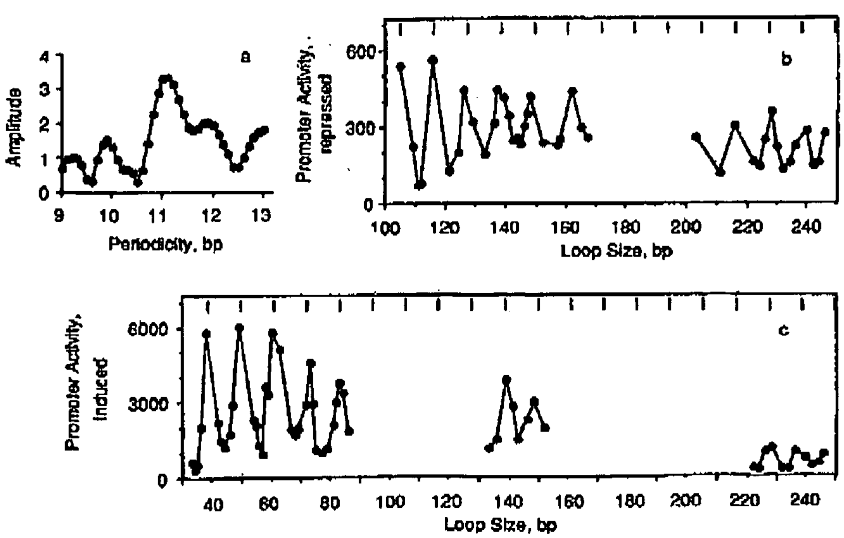

FIG. 3. Change of promoter activities with loop size as measured by the β-galactosidase assay. (*a*) Frequency spectrum of promoter activities computed by Fourier transformation. The 95 points shown in *b* and *c* were used in the analysis. In *b* and *c* the hashmarks at the top are spaced 11.1 bp apart. (*b*) Basal level of the *araBAD* promoter in strain DL100. (*c*) Induced level of the *araBAD* promoter in strain SH326 (*cya*$^-$ and elevated concentrations of AraC protein).

containing larger loops and retaining the CAP site. Even the small-spacing candidates with separations between *araO*$_2$ and *araI* of 34, 33, and 32 bp, which leave just the two AraC protein binding sites intact, remained repression-positive. Thus there is no lower limit to the *araO*$_2$–*araI* loops.

We also examined *araO*$_2$ involvement in forming these small loops. First, an *araO*$_2$ point mutant (G → A at position −271) was constructed and its effect on expression was tested in a 44-bp spacing plasmid. This mutation reduced repression by a factor of 2, showing that *araO*$_2$ is involved in the loop. We used occupancy of *araO*$_2$ as a second measure of loop formation in the small-loop plasmids. The affinity of *araO*$_2$ for AraC protein is insufficient for it to be occupied by AraC protein on its own. However, this site is occupied when it can loop to *araI* or *araO*$_1$ (4). *In vivo* footprinting of *araO*$_2$ showed it was unoccupied in the repression-minus plasmids with spacings of 38 and 49 bp but was occupied in the repression-positive plasmids with spacings of 33 and 44 bp, consistent with formation of the postulated small loops (data not shown).

Periodicity. Oscillations in repression ability as a function of spacing extend to about 400-bp loops. The hashmarks in Fig. 3 *b* and *c*, which are spaced 11.1 bp apart remain in phase with the oscillations for loops 40–240 bp long, implying that the helical repeat of the DNA *in vivo* between *araI* and *araO*$_2$ is 11.1 bp per turn. Fourier transformation of the same 95 points (Fig. 3*a*) objectively shows the same result.

Periodicity and Linking Number Deficit. The linking number deficit of *E. coli* DNA could tend to untwist the DNA. Therefore, we tested whether a reduction in DNA gyrase activity would alter the apparent helical repeat in the *ara* system. Twelve plasmids with 222- to 246-bp spacings between *araI* and *araO*$_2$ were transferred to strain DL110, which is deficient in gyrase activity. In experiments not shown, topoisomer analysis on chloroquine-containing gels of plasmid DNA extracted from these cells showed it to contain about half the linking number deficit as plasmid DNA extracted from the strain used in the previous experiments. The change in the apparent helical repeat in the *ara* system, however, was barely detectable, if present at all, perhaps 2–4 bp in the loop tested (Fig. 4).

Not having remeasured the entire set of spacing plasmids in the gyrase mutant strain, we cannot tell whether the nearest peak shifted 2–4 bp or some more distant peak shifted. To determine whether a more distant peak shifted by more than one cycle, we examined the apparent shift in loops of about half the size. We found a shift of at most 1 or 2 bp

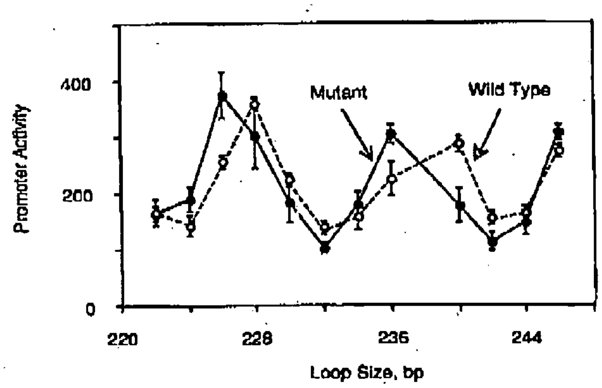

FIG. 4. Basal level of P_{BAD} in a set of insertion plasmids in wild-type strain SH322 (solid circles) and the isogenic gyrase mutant strain DL110 (open circles) derived from five independent cell growth experiments and measurement of the β-galactosidase activity. The points show the average values, and the error bars show the standard deviations of the measurements.

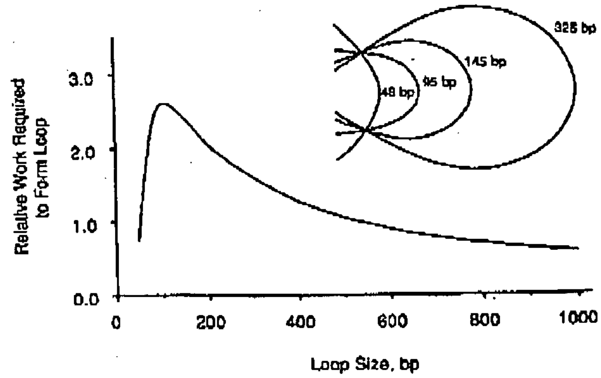

FIG. 5. Approximate shapes of looped DNA and the relative work required for the loop formation.

in a series of 11 plasmids with 121- to 148-bp loops, strongly suggesting that the shift in helical repeat in the gyrase mutant was no larger than 2 bp per 100 bp of loop.

Richardson *et al.* (11) analyzed changes in the helical repeat that were generated by varying the linking number deficit *in vitro*. They found a repeat of 11.07 for $\sigma = -0.061$ and a repeat of 10.95 for $\sigma = -0.031$ very similar to our *in vivo* finding.

Energetics of Loop Formation. The fact that very small loops can form when *araO*$_2$ and *araI* are correctly oriented suggests that AraC protein must bend significantly in the formation of these loops. Clearly, the actual looping process is aided by the supercoiling present *in vivo* (2, 31, 32), and the results of calculations of the energies depend on the physical model chosen for looping. Nonetheless, as a crude approximation, we determined the approximate relative work necessary to form loops of various sizes if the protein had a fixed diameter but was highly flexible (Fig. 5). As the loop size decreased, the work required for loop formation first increased, then declined. The decline was obtained when the protein bent appreciably to form the loop, ultimately reaching a region of somewhat dubious physical significance.

We cannot easily estimate the propensity to loop as a function of loop size from these considerations, since $K = \exp(-\Delta G/RT)$ and $\Delta G = \Delta H - T\Delta S$. We have estimated relative changes in H but cannot estimate ΔS for loop formation.

DISCUSSION

The L-arabinose operon of *E. coli* has long been known to be positively and negatively regulated by AraC (33). Binding AraC protein at *araI* and at *araO*$_2$, sites separated by 211 bp, and association of these two proteins to form a DNA loop is necessary for the negative regulation or repression of the *araBAD* promoter P_{BAD} (1–4). As DNA looping appears to be a wide-spread and versatile mechanism utilized in gene regulation, we have explored the parameters of looping in the *ara* system.

We found that separating *araI* and *araO*$_2$ by more than about 500 bp eliminated detectable repression. At the other extreme, no lower limit to their spacing was found, implying that AraC protein possesses significant flexibility. As the spacing between *araO*$_2$ and *araI* varies, the level of expression of P_{BAD} oscillates with a period of about 11.1 bp, suggesting that *in vivo* the DNA between *araI* and *araO*$_2$ possesses a helical repeat of about 11.1 bp per turn.

We are surprised at the relatively small upper limit to the *ara* loop size. In the *deo* system a small amount of looping is detectable when the relevant operator sites are separated by as much as 5000 bp (34). Similarly, the enhancers of eukary-

otic genes likely utilize DNA looping (35) and they often function from distances exceeding 500 bp. Certainly, however, as the distance separating *araI* and *araO₂* increases, looping must become more difficult because of the lowered concentration of one site in the presence of the other—i.e., for entropic reasons.

As a first approximation to estimating distance effects on looping, we might estimate the concentration of $araO_2$ in the presence of *araI* by assuming the intervening DNA was a long polymer with random angles between stiff segments. This is equivalent to a three-dimensional random walk (36), and yields concentration as a function of the distance ℓ between the sites varying as $\ell^{-3/2}$. If the loop size is increased from 300 to 500 bp, this relationship predicts a decrease in concentration of one site in the presence of the other by 54%. This result is at significant variance with the data presented in Fig. 2 in which the effective concentration must change by much more so that the repression changes from full to undetectable over this range. Presumably the estimation is poor because of the unknown orientation and position requirements of *araI* and $araO_2$ for looping in addition to the unknown effects of DNA supercoiling and binding by other proteins.

Our experimental result that there is no lower size limit for the *ara* loop suggests that AraC protein is flexible. This would have to be a particular flexibility however. On one hand the protein appears free to bend parallel to the DNA. On the other hand, the fact that misorienting *araI* and $araO_2$ interferes with repression implies that the protein is not significantly free to bend in a direction perpendicular to the axis of the DNA. If we assume the protein is completely free to bend in the direction required to form small loops and that it holds the *araI* and $araO_2$ sites a fixed distance apart, we can approximately determine the loop shape and the relative work required for formation (Fig. 5). As we have discussed, larger loops, while energetically more easily formed, are less favored for entropic reasons.

One interesting outcome of the looping energetics calculations is the apparently greater work required to form a loop of about 100 bp (Fig. 5). This maximum may correspond to the relatively poor repression seen for loops of 140–160 bp (Fig. 3*b*).

Our finding of the 11.1-bp periodicity of *ara* P_{BAD} repression raises the question of the structure of DNA *in vivo*. A number of experiments have shown that the helical repeat of linear DNA *in vitro* is about 10.5 bp per turn (6–8). Special sequences can give rise to different helical repeats—e.g., 10.0 ± 0.1 bp per turn with poly(dA)·poly(dT) and 13.6 bp per turn with poly(dG-dC)·poly(dG-dC) (37, 38), but sequence analysis of the *ara* P_{BAD} regulatory region reveals no special base distributions or sequence features with periodicities of 10–12 bp. Additionally, the independence of the oscillation cycle with various sizes of spacing and with various combinations of sequences by random deletions and insertions nearly excludes the possibility that the sequence alone of the *ara* regulatory region is responsible for the 11.1-bp periodicity we observed.

We suspect that the linking number deficit of *E. coli* DNA shifts the average helical repeat from its relaxed value of 10.5 bp per turn to around 11.1. A linking number deficit can generate a torsion that generates supercoiling. This same torsion is in a direction that it could also act to unwind the DNA. In fact, the apparent supercoiling density *in vivo* measured on a plasmid was appreciably less than its probable linking number deficit (9). If the remainder of the linking number deficit were applied to untwisting the DNA, the helical repeat could approach 11 bp per turn.

We thank Sharon Jenkins for constructing small size oligonucleotides insertion plasmids, and Pieter Wensink, Alan Brunelle, Li Huo, Jim Tobin, John Carra, Robert Lobell, and Guy Duffaud for discussions and comments on the manuscript. This work was supported by National Science Foundation and National Institutes of Health grants.

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Attorney Docket No.: 3021.15US02

HACKETT et al. Confirmation No.: 5310

Application No.: 10/758,237 Examiner: Sullivan, D.

Filed: January 15, 2004 Group Art Unit: 1636

For: TRANSPOSON-INSULATOR ELEMENT DELIVERY SYSTEMS

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

VERIFIED STATEMENT UNDER 37 CFR § 1.821(f)

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SUMMARY STATEMENT
(Privileged Communication)

MARY LOU OSTER-GRANITE
(301) 496-1383
mo96o@nih.gov

Release Date: **09/05/2003**

Application Number: **2 P01 HD032652-09A1**

WHITLEY, CHESTER B MD, PHD
420 DELAWARE ST SE, BOX 446
MINNEAPOLIS MN 55455

Review Group: **CHHD-C**
Developmental Biology Subcommittee

Meeting Date: **06/16/2003**
Council: **OCT 2003**
Requested Start: **01/01/2004**

PCC: **MRDD -MO**

Project Title: **Gene Therapy for Metabolic Disorders**

SRG Action: **Priority Score: 137 Percentile: 2.8 #**
Human Subjects: **E4-Human subjects involved - Exemption #4 designated**
Animal Subjects: **30-Animals involved - no SRG comments or concerns noted**
Children: **4A-Child representation unknown,scientifically acceptable**

Project Year	Direct Costs Requested	Estimated Total Cost
9	840,870	1,246,599
10	848,353	1,257,692
11	873,804	1,295,424
12	900,022	1,334,292
13	927,023	1,374,322
TOTAL	4,390,072	6,508,329

ADMINISTRATIVE BUDGET NOTE: The budget shown is the requested budget and has not been adjusted to reflect any recommendations made by reviewers. If an award is planned, the costs will be calculated by Institute grants management staff based on the recommendations outlined below in the COMMITTEE BUDGET RECOMMENDATIONS section.

SCIENTIFIC REVIEW ADMINISTRATOR'S NOTES

RESUME AND SUMMARY OF DISCUSSION: This revised application requests continued support for an ongoing program project focused on basic aspects of gene therapy for mental retardation disorders. It encompasses preclinical experiments in animal models of human inherited metabolic diseases, as well as developing and testing new molecular genetic approaches to gene therapy. Three projects are proposed to explore the use of lentiviral vectors in a new animal model of mucopolysaccharidosis type I and the effectiveness of *ex vivo* and *in vivo* transfection of hematopoietic stem cells; to genetically correct a *trans*-dominant cerebellar ataxia mutation in a spinocerebellar ataxia mouse model; and to explore a novel non-viral method of transgene integration based on a transposon system. An administrative office, a microchemical facility, an animal resource, a cell processing service, a real-time quantitative PCR core, a viral vector service, and a neurological facility support these projects.

This is a considerably improved application. The investigators have responded positively to concerns raised at the previous review. It offers a range of diverse approaches, complementary but conceptually unified by the common goal, with evidence of cross-communication and productive synergy, contribute to making this a highly meritorious application. The program covers aspects of gene therapy that approach clinical application, as well as basic aspects that more remove from therapy. Considerable challenges remain, but the proposed approach is innovative and its potential impact on the field is important. Although there are some comments concerning the structure of the core facilities, they are integral part of the program that provides excellent technical services. Overall, the application was recommended for further consideration for the time and amount requested with a priority score of 137.

DESCRIPTION (provided by applicant): The central theme of this program (revised application) is the development of innovative gene therapy strategies, especially for metabolic disorders causing mental retardation. The program is highly focused on identifying and resolving the barriers to clinical gene therapy. In the re-organized program, three projects aim to exploit recent innovations that will enhance gene delivery and expression. Project I, Lentiviral gene therapy for mucopolysaccharidosis type I (Whitley) builds upon characterization of a "true" murine knockout model of Hurler syndrome in which newborn treatment virtually "cures" the pathologic features. Project II, Gene therapy for cerebellar ataxia (McIvor) explores methods to inactivate a detrimental mutant gene in the brain. (Project III has been removed in response to the NIH Study Section critique.) Project IV, Sleeping Beauty transposon for gene therapy (Hackett) will evaluate a new, non-viral method of integrating therapeutic genes into mammalian chromosomes *in vivo*, aiming to optimize efficiency and safety. Institutional support for this program includes co-localization of project investigators in new, state-of-the-art laboratory facilities. These projects utilize a number of models of human metabolic disorders causing brain disease, notably, rodent models of mucopolysaccharidosis, ornithine transcarbamylase deficiency, and spinocerebellar ataxia. The program shares core facilities for administration (Whitley), microchemical synthesis and analysis (Brown), real-time quantitative PCR (Pan), hematopoietic stem cell processing (Miller), animal resources (Gillett), viral vector production (McIvor), and neurological procedures and analysis (Low).

CRITIQUE NOTE: The sections that follow are the essentially unedited, verbatim comments of the reviewers assigned to this application. They are provided to illustrate the range of opinions expressed. The application was discussed and scored by all reviewers present. The attached commentaries may not necessarily reflect the position of the reviewers at the close of group discussion, nor the final majority opinion of the group. The Resume and Summary of Discussion as well as the Overall Critique, however, are the authoritative representation of the final outcome of group discussion.

RESEARCH PROJECTS

PROJECT I LENTIVIRAL GENE THERAPY FOR MPS TYPE 1
(Chester Whitley, Ph.D., M.D. & Nikunj Somia, Ph.D., pp. 200-245)

DESCRIPTION (provided by applicant): Previous experience with clinical bone marrow transplantation has shown that normal marrow is capable of providing life-long systemic metabolic correction for patients with MPS diseases; however, translation of this principle to *ex vivo* hematopoietic stem cell gene therapy has been limited by: (a) the inability of MLV-based retroviruses to integrate into non-dividing cells; (b) high-titer vector preparations to achieve a substantial multiplicity of infection (MOI); and (c) immunologic selection against transgene-expressing cells. However, recent development of third generation lentiviral vectors suggests an approach that may obviate these problems with broad implications for many applications of *ex vivo* hematopoietic stem cell gene therapy.

The overall objective of this project is to accomplish the requisite preclinical studies for *ex vivo* hematopoietic stem cell gene therapy for mucopolysaccharidosis type I (that is, Hurler syndrome and variants). Toward this objective, Specific Aim 1 will construct HIV based lentiviral gene transfer systems that will be useful for treatment of human neuropathic conditions represented by the MPS diseases, and particularly targeted at transduction in human hematopoietic stem cells. Specific Aim 2 will evaluate the potential for intravenous administration as a means of *in vivo* lentiviral bone marrow stem cell transduction and metabolic correction, as well as the potential for *in vivo* selection of transduced cells. Follow-up evaluations will study the response to genetically-modified hematopoietic stem cells, specifically determining the level of recombinant MPS enzyme activity achieved, the duration of expression, and clinical response. Toward an initial clinical trial, Specific Aim 3 will study vectors for expression of the normal IDUA human enzyme delivered by *ex vivo* lentiviral transduction of granulocyte colony stimulating factor (G-CSF) mobilized $CD34^+$ human peripheral blood progenitor cells after engraftment into *NOD/SCID* mice. In phases of this study, more complex bicistronic message vectors will be studied for the ability to enhance IDUA gene expression by means of *in vivo* selection of transduced cells based on antifolate drug resistance). Aliquots of G-CSF mobilized $CD34^+$ human peripheral blood progenitor cells will be treated with IDUA vector, or IDUA-DHFR vector, and studied for (a) transduction of colony-forming cell (CFC); (b) transduction of long-term culture initiating cells (LTCIC); and (c) transduction of marrow-repopulating cells in *NOD/SCID* mice. These studies are directly aimed at developing a clinical trial of *ex vivo* hematopoietic stem cell gene therapy for the model disease MPS I. However, the studies will have much greater significance for the broader application of lentiviral vector systems and especially for application in hematopoietic stem cell gene therapy.

CRITIQUE 1:

SIGNIFICANCE: These studies are chiefly aimed at developing a clinical trial for *ex vivo* hematopoietic stem cell (HSC) gene therapy for the model disease mucopolysaccharidosis type I (MPS I). However, the studies might have greater significance for the broader application of lentiviral vector systems, especially for HSC-mediated gene therapy. The specific disease to be addressed – α-L-iduronidase (IDUA) deficiency (Hurler syndrome) and its MPS I variants - is important in its own right and has not been satisfactorily addressed by present approaches. In addition, the MPSs have historically served as prototypes for other lysosomal storage diseases. Therefore, there is significance for the gene therapy field in general.

Previous experience with clinical bone marrow transplantation (BMT) has shown that normal marrow is capable of providing life-long systemic metabolic correction for MPS patients. However, optimization and extension of these principles to *ex vivo* manipulation of HSCs for gene therapy has been limited by some of the problems to be addressed in the present application: (1) the inability of murine leukemia virus (MLV)-based retroviruses to integrate into non-dividing cells has limited the efficiency of engineering HSC – which are difficult to expand and maintain in the cell cycle *ex vivo* – for the treatment of metabolic diseases via BMT. Lentiviral vectors, which can deliver a transgene across the nuclear membrane of non-mitotic cells and infect quiescent stem cells, appear to circumvent this limitation. (2) Another limitation to viral vector-mediated gene therapy is the present difficulty in

obtaining high titer vector preparations to achieve a substantial multiplicity of infection (MOI). This problem will be approached in this application through stabilization of the vector particle with vesicular stomatitis virus G envelope protein or VSVG (via co-transfection) allowing for concentrations of 1,000-fold greater than presently available. (3) There is a risk of immunologic selection against transgene-expressing cells. If this application is successful in demonstrating that 3rd generation lentiviral vectors address some of these problems, then it will have broad implications for many applications of *ex vivo* HSC gene therapy.

There is a great deal of focus on working out the fine non-flashy but pivotal details of lentiviral infection of HSCs for ultimate clinical trial. This is groundwork that needs to be done to make the therapies possible (i.e., the devil is in the details). This has practical significance in expediting progress towards a clinical trial.

APPROACH: The proposed work will focus on the use of lentivirus as a gene delivery vehicle to the HSCs of the recently generated α-L-iduronidase (IDUA)-deficient mouse model of MPS I. The overall objective is to accomplish the requisite preclinical studies for an ultimate clinical trial of *ex vivo* HSC-mediated gene therapy for MPS I. This project seeks to test the hypothesis that the application of HIV-based lentiviral vector gene therapy has a greater potential for gene delivery to HSCs than other viral vectors. The current funding period's work was done on a surrogate model, the MPS VII mouse; however, the recent derivation of the new MPS I mouse – albeit not as well characterized as the MPS VII mouse – has prompted the investigators to shift – rightly so – to this model for the project's renewal application.

The hematopoietic stem cell to be manipulated are either derived from the animals endogenously (via intravenous – tail vein – administration) or exogenously to be manipulated *ex vivo* with lentivirus and then reimplanted into the animals. In twelve studies, divided across three specific aims, the investigators proceed to delineate a number of very detailed, incremental steps in fairly well defined murine experiments. The overriding goal appears to be to accumulate enough preclinical data to be able to move onto clinical trials. To that end, a series of very specific questions are addressed – e.g., proper titer, proper temperature, assessing and trouble-shooting each phase of viral transduction (attachment and penetration, integration, and expression), etc.

Specific Aim 1 will explore a new 3rd generation lentiviral vector *in vitro*. The target cells to be examined are 293T (human) cells, murine IDUA-deficient fibroblasts, human IDUA-deficient fibroblasts, and MPS I patient $CD34^+$ (HSC) cells.

To study retroviral-mediated gene therapy, ten different MLV-based vectors were constructed to express IDUA from various promoters and in various constructions. To evaluate the relative levels of transgene expression, the vectors will be used to transduce $CD34^+$ cells from patients with MPS I, and the cells will then be put into "long-term culture initiating cell assays" as an assay for hematopoietic progenitors. In response to reviewers' recommendations from the previous round, one of these vectors was chosen for more extensive characterization (lentiviral vector CS-PI) in a hollow-fiber bioreactor system. Also in Aim 1, the investigators will stabilize the vector particles with VSVG envelope protein to allow for concentration 1,000-fold. They will also use VSVG pseudotyping to alter the target cell specificity.

Aim 1 includes the following five studies: Study 1: construct vectors; Study 2: develop Q-PCR assays to evaluate vector transduction, transgene expression, and biosafety; Study 3: dissection of the lentiviral vector pathway by looking at attachment and penetration, integration (via PCR amplification of the integrated vector), and expression (number of GFP-expressing clones); Study 4: the effect of MOI, vector, cell concentration, and temperature; and Study 5: development of a stable producer cell line for 3rd generation lentiviral vectors.

Specific Aim 2 seeks to examine the transduction ability of the vector *in vivo* on endogenous bone marrow-derived HSCs of the MPS I mouse following intravenous (tail vein) injection, as well as the potential for *in vivo* selection of transduced cells. Evidence of metabolic correction will be examined by assessing the following: (a) level of recombinant MPS enzyme activity achieved, quantitation of α-L-iduronidase enzyme activity, expression of gene by RT-PCR, possible immunoreactivity of the enzyme (if the newly derived polyclonal antibody works); (b) duration of expression; and (c) clinical response (e.g., motor and cognitive tests). Further evidence that stem cells have been transduced will be obtained by serial BMTs, i.e., transplantation of marrow from vector-treated MPS I mouse into a second generation of MPS I mice. This is an important step for defining a true stem cell and is applauded by this reviewer.

Aim 2 encompasses the following three studies: Study 6: evaluation of the IV administration of MPS mice with the 3rd generation lentiviral vector CS-PI expressing IDUA; Study 7: determine the biologic effect of adding a protein transduction domain to the native IDUA protein – in other words, evaluation and comparison of IV administration of 3rd generation vectors expressing IDUA alone with those expressing a fusion protein with a TAT sequence that will facilitate entrance of proteins into mammalian cells; Study 8: assess the potential for *in vivo* selection of dihydrofolate reductase (DHFR)-expressing vector/cells by administering methotrexate. In these studies, the following parameters will be assessed: FACS analysis, quantification of IDUA enzyme activity, molecular genetic analysis of gene transfer via real-time quantitative PCR, quantification of urinary glycosaminoglycan (GAG) excretion, localization of IDUA protein expression, pathological examination, and behavior studies.

Specific Aim 3 will examine the *in vivo* effects on the histology and enzyme expression of mice following the transplantation of HSCs (G-CSF mobilized-$CD34^+$ cells) that have been transduced *ex vivo* with this same vector. *NOD/SCID* mice will be transplanted with human $CD34^+$ cells. Some of these peripheral blood progenitor cells will be obtained from MPS I patients transduced *ex vivo*. These cells will be examined *in vitro*. Long-term cultures will be established by co-culturing 10,000-50,000 of these cells with M2-10B4 in collagen-treated transwell inserts. Then the project will move to its *in vivo* phase. Some animals will receive serial transplants. The degree of metabolic corrections and phenotypic correction will be assessed. In phases of this study, more complex bicistronic message vectors will be studied for the ability to enhance IDUA gene expression by means of *in vivo* selection of transduced cells based on antifolate drug resistance. Therefore, aliquots of G-CSF-mobilized $CD34^+$ human peripheral blood progenitor cells will be treated *ex vivo* with an IDUA vector *or* IDUA-DHFR vector. To assess the potential for selection of transduced cells *in vivo*, one group of *NOD/SCID* mice will, therefore, receive methotrexate. The end-point to be examined will be an increased number of IDUA-transduced cells measured by quantitative PCR and RT-PCR.

This aim includes the last three of the 12 studies: Study 9: evaluate IDUA vectors following *ex vivo* transduction of HSC and subsequent growth *in vitro*, and optimize a clinically-applicable standard operating procedure; Study 10: assess *ex vivo* transduction protocol by determining engraftment in *NOD-SCID* mice; Study 11: assess *ex vivo* transduction protocol by transduction of IDUA-deficient murine HSCs, with subsequent transplantation into IDUA-deficient mice (now combined with Study 10); and Study 12: assess the potential for *in vivo* methotrexate (MTX) selection of DHFR-expressing vector/cells.

Prior criticisms of this application from the previous review have been responded as follows: Criticism regarding the lack of a technique for measuring presence of the IDUA protein that will be critical for analyzing cells and animal tissues for the efficiency of IDUA gene expression is being addressed by attempting to generate an antibody against human IDUA. The specificity of a polyclonal antibody is being evaluated now, though the investigators point out that species-specificity in the IDUA-deficient mouse is less of a concern. This criticism seems to have been addressed adequately.

Concern was raised that there was no mechanism in place – either in the transgene and/or the infected cell and/or the vector – to turn off the gene and/or eliminate infected cells or the virus (that is, a suicide gene) once *in vivo*. The investigators' response was, in essence, that no one does this and it is not necessary at this point. This is probably not a satisfactory response and the investigators should – if not in the initial studies, then in future studies – consider how they will deal with adverse affects from the integrated vector and/or gene itself.

Regarding the paucity of detail as to exactly which approaches will be used – e.g., for pseudotyping, etc. This concern is now adequately provided.

Some persistent concerns nevertheless remain. Two mice per experimental condition are simply inadequate and are not likely to be informative. A power analysis should be done to determine how many animals are necessary for statistical significance and the research plan adjusted accordingly. Despite the fact that MPS I does have a neurological phenotype and that HSCs may pass through the blood-brain barrier, the application still pays inadequate attention to detailing the kind of cells that will be altered in the brain following lentiviral injection - nerve cells? Microglia? However, there is a good Neurological Services Core that will probably be able to look into this aspect of the application.

INNOVATION: This study uses the newly-generated MPS I mouse; the intravascular delivery of lentivirus to infect endogenous HSCs (an unexpected result from a safety study); the use of pseudotyping to allow for better concentration of the virus to achieve higher titers; the use of TAT coupled to IDUA to improve cell uptake; and the use of *in vivo* selection of infected HSCs via methotrexate.

INVESTIGATOR: The principal investigator, Dr. Chester Whitley, is Professor of Pediatrics and Human Genetics. Dr. Whitley is a leader in this field for many years. He has been productive, and is quite capable of directing this project. The co-investigator, Dr. Nikunj Somia, is Assistant Professor of Genetics, Biology and Development, and Human Genetics. His expertise is lentivirus biology, vector design, and vector-cell interactions.

ENVIRONMENT: The resources at University of Minnesota in Minneapolis are excellent for the studies proposed. The project is also well supported by the program's core facilities.

OVERALL EVALUATION: This competitive renewal application proposes to study the efficacy of 3rd generation lentiviral vectors to infect and manipulate HSCs for increasing their expression of IUDA, the enzyme deficiency in MPS I. The investigators plan to delineate the process of lentiviral infection and gene expression in a very logical, step-by-step, workman-like fashion. The systematic approach proposed will likely troubleshoot the system adequately such that usable vectors will be generated by the end of the granting period. The project will rely on the facilities at the Cell Processing Core and the Neurological Services Core.

CRITIQUE 2:

SIGNIFICANCE: Therapeutic interventions using bone marrow transplant on mucopolysaccharidoses diseases have shown a modest success. While peripheral effects on most organ systems are observed there has been a failure to achieve major therapeutic benefit in the CNS. The hypothesis is that HSC transduced with a gene therapeutic product will access the CNS compartment through differentiation into microglial cells capable of expressing the gene product. Since lifetime engraftment of HSC are necessary, retroviral vectors are proposed to be used expressing MPS I. These studies in animal models if successful are the basis of a potential human clinical trial using bone marrow stem cells transduced by lentiviral vectors transduced by MPS I.

APPROACH: The project is composed of three highly refined specific aims that are logical in as they

been highly scrutinized in two prior reviews and the principal investigator has done his best to address the concerns and potential flaws of this specific project. Specific Aim 1 needs to address concerns raised at the previous reviews: what is the vector of choice? How will it function? What will finally transduce HSC? Another reservation is when expressing IDUA enzyme in cells how will one know that it is expressing and where? Finally, the issue of turning on/off gene expression as well as of eliminating the transduce cells are potentially critical issue. The investigators have done a very good job detailing the strategy of what vector type and what eventual vector configuration they will ultimately use to transduce the stem cells. They have more than significant expertise to choose what the vector will be, what the envelope will be, etc. The key in these experiments will be duration of expression of these vectors in conjunction with expression in stem cells when differentiated into monocytes. The concern that a histochemical/antibody assay was not available is still an issue. While progress has been made in identifying a peptide sequence that was potentially immunologic, no antibody yet exists. While use of laser capture technology with quantitative real-time polymerase chain reaction (QRT PCR) is feasible, the robustness and throughput of the assay is critical for clinical development as well as large animal studies. The final critique is probably more of a protocol/clinical concern. Two recent reports on the use of murine retroviral vectors with HSC noted two leukemia adverse advents. While the project will focus on preclinical models, it would seem that the desire to move to human trials would be beneficial to develop a class of regulated or eliminable vectors.

In Specific Aim 2 the major critique was the MPS mouse knockout and availability of significant quantity of animals. The pathophysiology of the mouse model seems to simulate the human condition and is probably similar to Hurler syndrome. The fecundity of the mouse colony now appears adequate to provide the animal numbers to perform the proposed revised studies.

Finally, Specific Aim 3 was not highly critiqued in prior submissions and still seems logical on the basis of the revised Specific Aims 1 and 2.

INNOVATION: This project makes use of the MPS I knockout mouse, which is a model for Hurler syndrome. The use of lentiviral vectors and stem cells for replacing an enzyme defect is novel. So is the delivery of lentiviral vectors to endogenous HSC.

INVESTIGATOR: Dr. Whitley is an expert in the field of genetic diseases involving mucopolysaccharides. He has assembled a group of first class investigators to assist in this project as well as the program. In particular, Dr. Somia is an expert on retroviral vectors and capable of directing the vector aspects of the project.

ENVIRONMENT: The overall facilities and interactions of the investigator at University of Minnesota are excellent. The cores will support the project. Institutional commitments are also adequate.

OVERALL EVALUATION: This project is certainly well thought out, organized, and has a higher than average chance of success. The investigators have an excellent track record of both innovative basic as well as translational clinical research. While key issues such as antibodies to IDUA remain to be achieved, excellent alternatives exist. The area of concern is the perceived safety of using lentiviral vectors with HSC. While not critical for this project, this issue must be answered for eventual entry into early clinical trials.

CLINICAL RESEARCH: Donated tissue specimens will be curtured *in vitro* or administered to mice

RECOMMENDATION: Project I is recommended for further consideration as requested with a priority score of 169.

PROJECT II GENE THERAPY FOR ATAXIA

DESCRIPTION (provided by applicant): The cerebellum is a region of the brain that plays a major role in the control of movement and cognition. Spinocerebellar Ataxia Type 1 (SCA1) is a fatal human disorder that is one of at least five inherited ataxias, and a total of at least eight neurodegenerative diseases, each caused by the expression of a mutant protein containing an expanded polyglutamine region. Pathogenesis is due to a gain of function conferred by mutant ataxin-1 protein and has been recapitulated in a transgenic mouse model resulting in degeneration of neuronal Purkinje cells in the cerebellum. As a potential approach toward gene therapy for SCA1, the investigators demonstrated during the current period of study: (i) that adeno-associated virus vectors (AAV) are an effective tool for *in vivo* gene transfer into cerebellar Purkinje cells, yielding up to 3% transduction in a cerebellar hemisphere after a single intracerebellar injection; and (ii) that ribozymes and antisense RNAs targeting specific positions in the ataxin message destabilize ataxin message *in vitro* and in tissue culture cells *in vivo*, respectively.

In this competing renewal application, the investigators propose to extend these studies through the execution of four aims. Specific Aim 1 will further characterize and optimize AAV-mediated Purkinje cell transduction *in vivo*, including the molecular role of basic fibroblast growth factor receptor 1 and heparin sulfate proteoglycan in virus binding, and the relative effectiveness of AAV serotypes 1, 2, and 5 in mediating transduction of cerebellar Purkinje cells. Specific Aim 2 will continue testing molecular approaches for downregulating or correcting expanded ataxin-1 message, including antisense, ribozyme, *trans*-splicing and RNA interference. Specific Aim 3 will establish antiataxin transgenes, subsequently crossing with SCA1 transgenic mice to evaluate the effectiveness of these approaches in controlling Purkinje cell degeneration and development of ataxia. Specific Aim 4 will use AAV vectors to deliver anti-ataxin or ataxin correcting sequences to cerebellar Purkinje cells in *SCA1* transgenic mice, as a direct model of gene therapy for SCA1 in humans. Results from these studies will thus provide optimized molecular tools and delivery conditions that will be applicable to treatment of not only SCA1 but other dominant-acting and other neurologic disorders as well.

CRITIQUE 1:

SIGNIFICANCE: Spinocerebellar Ataxia Type I (SCA1) is an important, fatal, untreatable human disease, one of the inherited ataxias. Devising therapeutic strategies, however, will have significance beyond this disease; it may also inform the broad family of CAG trinucleotide repeat/expanded polyglutamine gain-of-function neurodegenerative diseases, of which Huntington's disease and spinobulbar muscular atrophy are other members. The attempt to ameliorate abnormal gene action at the level of translation could prove to be very important. Most gene therapy paradigms to date have dealt principally with supplying a missing or essential gene product. This application could potentially offer insights into broader applications of gene therapy approaches, particularly to the very challenging task of inhibiting – not augmenting – expression of a gene, in this case *SCA1*.

APPROACH: While the investigators were quite sloppy in renumbering the figures in this revision and simply tried to shoehorn their changes into their pre-existing draft, and while the figures had no legends – all combining to make reading this application difficult – they do seem to have addressed some of the prior concerns.

The investigators wish to use AAV to alter gene expression in a SCA1 mouse model. The aims, while quite ambitious and labor-intensive, do flow logically from one to the other and constitute an appropriate approach for determining whether gene therapy (particularly via AAV) is a reasonable approach to such diseases. They have chosen AAV – rather than lentivirus as previously suggested by one of the reviewers – because of their extensive experience with this vector and their relative success to date with this vector in the model to be studied. This reviewer agrees, but feels that the investigators should nevertheless consider lentivirus if spread of the designated transgene or if its expression is greater than that achieved to date with AAV.

Specific Aim 1 proposes to optimize AAV-mediated transduction of the cerebellum by comparing the efficiency of various AAV serotypes – AAV-2 (the type used in preliminary studies with a 3% efficiency in transducing Purkinje cells stably for a year) *versus* AAV-1, AAV-4, and AAV-5. While acknowledging that altering the serotype may also alter the range of cell types that can be infected and therefore introducing another level of complexity, the investigators indicate that they will focus entirely on transgene expression in Purkinje cells and use that alone as a read-out of the relative value of a given serotype for this particular disease. They will also (i) determine the genetic form (episomal, integrated, or concatemer) of the AAV that supports long-term expression best in Purkinje cells. This analysis will be accomplished by various real-time quantitative PCR reactions for the GFP transgene. (ii) Further pursue the hypothesis the basic FGF receptor type 1 (bFGFR1) is pivotal for mediating AAV transduction of Purkinje cells. This type of assessment would be quite easy if cultures of Purkinje cells could be established efficiently. This however does not appear to be the case. Hence these studies must be performed *in vivo,* and to be accomplished by blocking bFGFR1 *in situ* and determining whether transduction efficiency has been blunted. Blocking will be done by either (a) pre-injecting a neutralizing FGFR1 antibody into the AAV injection site (using an irrelevant antibody – anti-tyrosine hydroxylase as a control) or (b) by infusing bFGF itself to block the receptor. (iii) Finally, the investigators will attempt to determine the role of heparin sulfate proteoglycans (HSPG) in mediating AAV transduction. Interestingly, heparin is a necessary co-factor for bFGF action. They will test whether HSPG is a receptor for AAV-mediated transduction by infecting the cerebellum of the MPS I mouse in which HSPG expression levels are inherently altered (a very ingenious approach). Infection could be diminished in these animals. However, because a rationale could also be made for increased transduction – for example, if excessive accumulation of matrix forces the AAV to concentrate on the cell surface – HSPG could also be plausibly implicated with the opposite result. The only outcome that would compellingly rule out a role for HSPG would be "no difference" in the MPS I mouse, which might be difficult to ascertain convincingly. This point was raised in this application's initial review. The investigators simply seemed to sidestep this concern, providing an answer that is difficult to understand, unresponsive, and unhelpful. Nevertheless, it is an interesting approach and is not a fatal flaw, given that it is not a key part of this application.

Specific Aim 2 proposes to determine which of four techniques for ataxin-1 blocking is most effective *in vitro*. The techniques are (1) anti-sense, (2) ribozyme, (3) *trans*-splicing, and (4) RNAi. The major contributions for this renewal will be the latter two. The investigators propose to develop a *trans*-splicing strategy for molecular repair of the expanded ataxin-1 message. This approach will be compared with the prior approaches used by the investigators during the first funding period – antisense and ribozyme RNA techniques that reduced the steady-state levels of ataxin-1 mRNA and protein in cultured mammalian cells. The *trans*-splicing strategy seeks to repair rather than eliminate the ataxin-1 message, making this attractive because it inhibits expression of the aberrant message while preserving normal expression of the gene, which will require assaying by PCR technology since simple immunocytochemistry for protein presence will not be adequate. On the other hand, RNAi vectors will be constructed based on insights derived from the most effective anti-sense constructs used previously. The methods proposed seem quite reasonable and state-of-the-art. The most effective ataxin-targeting sequences identified in Aim 2 will then be used to make the transgenic animals in Aim 3 and design the transgenes to be encoded by the AAV vectors in Aim 4.

Specific Aim 3 will essentially attempt to determine what the "gold standard" would be for gene therapy by creating a transgenic mouse in which normal ataxin-1 is expressed in every Purkinje cell of the SCA1 mouse cerebellum. This will be accomplished by generating a transgenic mouse expressing in its Purkinje cells the most effective ataxin-targeting sequences identified in Aim 2, subsequently breeding those transgenic mice with mice bearing the SCA1 gene. These animals will then be assessed histologically and functionally (both by motor and cognitive tests). If such a situation (which can only be achieved by transgenic technology) is insufficient to "rescue" the ataxic phenotype, then gene therapy will never prove efficacious under any circumstances. On the other hand, if the above-described murine progeny are asymptomatic, then the feasibility of gene therapy exists. The focus can

then legitimately be placed on optimizing transduction of as many Purkinje cells as possible with stably expressing corrective genes – the purpose of the application.

Aims 1 and 2, in that case, set the stage for Specific Aim 4, which is to generate AAV vectors bearing ataxin-targeting sequences characterized in Aims 2 and 3, of a serotype shown most efficacious in Aim 1, and then testing these vectors for their effectiveness in correcting the motor and cognitive dysfunctions as well as histological abnormalities of SCA1 after administration it into the cerebellums of SCA1 transgenic mice. This approach flows in a very logical manner and all aims seem critical for optimizing potential AAV therapies of SCA1. While a good deal of work is entailed, much groundwork has already been laid, including generating some of the transgenic mice. Also, this project will nicely take advantage of most of the program's cores (especially the Transgenic Core, the Vector Core, and the Neurological/Behavioral Testing Core).

It was suggested during the last review that an additional parameter to address might be the developmental state of the cerebellum at the time at which therapy is instituted. It was pointed out, for example, that delivering AAV to SCA1 mice at much earlier developmental stages than proposed, e.g., *in utero*, when Purkinje cells are being formed, may not only be a "poor man's" version of a transgenic animal (Aim 3), but optimize integration of a therapeutic transgene into a maximum number of Purkinje cells. In other words, it is likely that more cells will be infected and perhaps corrected. One could, in fact, perhaps see a "dose-response" of sorts – the more cells infected (earlier in development), the greater the histological and behavioral response. The investigators appeared to welcome this suggestion but made an error in indicating that they would institute therapy in the "newborns". The neonatal time point is probably too late for the effect described above and likely offers no advantage over treating juveniles or adults. To alter Purkinje cells during their genesis, the investigators will need to inject into the fetal cerebellum, which is a feasible intervention.

INNOVATION: There are a number of innovative aspects to this application. One of which is to approach a class of diseases – i.e., those without a clear-cut absent gene product but rather a gain-of-function mutation such as SCA1 – with a gene therapy approach. The use of a mouse with heparin sulfate binding abnormalities (MPS I) is an innovative way of approaching the question of whether HSPGs play a role in AAV infection. Similarly, examining the role of bFGFR1 receptor has not really been approached in the past, yet has been a matter of speculation. Creating a transgenic mouse in which all target cells are corrected as a proof-of-concept for gene therapy and as a "gold standard" to which gene therapy should strive is novel as well. Obviously, if correction of all cells with transgenic technology does not alter symptomatology, then it makes no sense to pursue a gene therapeutic approach at all.

INVESTIGATOR: This project will be directed by Dr. Scott McIvor, with support from Drs. Walter Low and Harry Orr, all affiliated with University of Minnesota, Minneapolis. Dr. McIvor is Professor, Institute of Human Genetics and Director, Gene Therapy Program. Dr. Walter Low is Professor of Neurosurgery and Physiology as well as Program in Neuroscience. Dr. Orr is Professor of Pathology and Biochemistry, and Director of Institute of Human Genetics. In addition, Dr. William Kaemmerer, Principal Scientist at Medtronic, Inc. in Minneapolis, will collaborate on this project as well. Together, this investigative team is well suited for this study and has made acceptable progress during the first two years of this project. Their preliminary data and techniques seem sound and well executed.

ENVIRONMENT: The resources and support are excellent and well suited to the goals of this project.

OVERALL EVALUATION: While ambitious and labor-intensive, this project does enumerate a series of specific steps (cast as individual aims) that flow quite logically one-to-the-other and are *de rigueur* for determining the feasibility and optimal construction of an AAV vector for addressing SCA1, a prototype for a gain-of-function disease, particularly related to polyglutamine repeats.

Previous reviews suggested that the experiments outlined here are far from being translatable to human therapies. For example, distribution of a transgene via AAV in a mouse brain will not readily translate to the larger expanse of the human brain. The investigators acknowledge this and indicate that they would, in subsequent years, extend the insights from this project to large animal models (though none really exists for cerebellar degeneration). This reviewer accepts this reasoning and believes that the importance of generating a practical gene transfer technique for addressing this class of diseases – heretofore unaddressed at even a proof-of-principle manner *in vivo* for any true animal model – outweighs the concerns for ultimate translation at this point. These experiments are important. In particular, various gene inactivating, blunting, or modifying techniques (RNAi *versus* antisense, ribozyme, or *trans*-splicing) will be compared head-to-head, looking at the same gene in the same cell type in the same CNS structure in the same animal model with the same fairly stereotypical and easy-to-monitor cytoarchitecture and functional read-outs (one of the virtues of looking at cerebellum). In this regard, it is appreciated that the investigators have continued to develop their aims since the last review. For example, the addition of RNAi (supported by preliminary data and predicated on insights from the anti-sense work) and the further development of the *trans*-splicing technique are noted.

Getting more Purkinje cells infected than the relatively small number shown under preliminary data will clearly be important in order to have a plausible impact on the disease. One will most likely need at least 10% of the Purkinje cells corrected (the point at which cerebellar symptoms usually become apparent) and perhaps as many as 50%. The investigators seem to be starting at 3% based on their best preliminary data. So they have their work cut out for themselves. It remains a challenge. Moving to earlier points in development – or at least seeing how that variable will impact the number of Purkinje cells infected – will be quite pivotal. Attempting to affect gene expression earlier in cerebellar development (including prenatally) may offer a greater chance of success in addition to providing important information on the evolution of the disease, defining therapeutic windows, and providing a "dose-response" curve.

CRITIQUE 2:

SIGNIFICANCE: Therapeutic intervention in genetic diseases involving triplet repeats is limited. SCA represents such a disease. Furthermore, the vast majority of corrective neurodegenerative research has focus on the cerebrum; efforts on cerebellar disease have been limited. This project proposes to take advantage of several systems known to inhibit gene expression combining it with gene transfer technology and testing in a mouse model system to validate the approach. The application is straightforward, and it should yield insight on this type of molecular strategy in general.

APPROACH: This resubmitted application has adequately addressed the majority of the concerns raised at the prior review. Three major issues were cited. The first involves the use of the gene delivery system AAV and alternative serotypes as well as potential synergy with lentiviral vectors used in Project I. The investigator clearly states the methods, reasons, and logical progression on how the vectors will be progressed for *in vivo* gene transfer. Transduction frequency and methods to address AAV use *in vivo* are addressed. Finally, while lentiviral vectors are available, the investigators reason that staying focused with AAV on a difficult project is commendable.

The second major concern was the use of the transgene that would be use to downregulate ataxin-1 message. The investigators, while experienced in antisense and ribozymes, were encouraged by reviewers to consider RNAi. The application now has added this element incorporated. The investigators' expertise should allow them the flexibility to evaluate all possibilities.

The third major concern was the overly ambitious nature of the project, involving generating transgenic animals with the appropriate ataxin target with mice bearing the SCA gene. Again, the timing seems appropriate given the core support and infrastructure of the University. The key experiment will be identifying the taxin target and generating transgenic mice, which are straightforward. Finally, the

investigators will put together the knockdown AAV vectors and test the target *in vivo*. These experiments are certainly worthwhile, and have implications in other genetic disease systems.

INNOVATION: The project is important not only for the specific disease to be addressed but for helping to determine whether diseases that need gene expression modification rather than just augmentation are amenable to gene therapy.

INVESTIGATOR: Dr. McIvor is an experienced and excellent investigator capable of carrying out the work described. In addition, he has an extremely talented set of collaborators for the project.

ENVIRONMENT: Dr. McIvor has excellent interactive collaborators with first class facilities at University of Minnesota, Minneapolis.

OVERALL EVALUATION: This is an excellent application, which is innovative, focused, and scientifically sound. Success of these integrative approaches may have implications beyond those of just this project. The combination of a strong project with excellent investigators makes this an outstanding project.

RECOMMENDATION: Project II is recommended for further consideration as requested with a priority score of 149.

PROJECT IV **SLEEPING BEAUTY TRANSPOSON FOR GENE THERAPY**
(Perry Hackett Ph.D.; pp. 500-537)

DESCRIPTION (provided by applicant): New non-viral gene transfer procedures are needed for human gene therapy in order to achieve long-term maintenance and expression of newly introduced genes. The purpose of this project is to evaluate the *Sleeping Beauty* (SB) transposon system for its efficacy to catalyze integration of several transgenes into selected tissues in mice and rats. The SB system is binary, consisting of a transposon containing a transgene and a source of transposase enzyme. The SB transposase is able to mediate enhanced integration of marker/reporter genes in several mammalian culture cell lines *in vitro* as well as in fertilized embryos of zebrafish and *Xenopus*. Here, the investigators propose to extend these studies in order to determine the efficacy of the SB transposon system as a gene transfer vector. Preliminary results indicate that the transposon can be delivered to livers of adult mice for long-term expression using hydrodynamic pressure. This procedure may not be possible to use in humans. Accordingly, the investigators will evaluate the efficiencies of alternative delivery of the SB system to livers of whole animals affected by single gene disorders that mimic conditions of human disease. Furthermore, as an additional precaution to curtail potential long-term effects of SB transposase, the investigators will examine two strategies to curtail transposase activity. They will concentrate on gene therapy for mucopolysaccharidosis. Thus, the aims of this project are to: 1) construct safer transposon vectors with either insulator elements, immediately inside both ends of the transposon vector, to keep the enhancers driving a transgene from activating neighboring genes or that contain a "suicide gene". 2) Determine the efficiency of SB for gene transfer and expression in the livers of mice; SB will be tested as a vector system for gene transfer into the liver using a transposon engineered to express genes whose activities are deficient in certain human diseases. 3) Determine the efficiency of SB for gene transfer and expression in the livers of mice using purified SB transposase rather than its gene; to assure limitation of transposase activity, and the efficacy of using purified SB transposase accompanying transposons will be tested in liver cells. The experiments in this project will provide an assessment of the capacity of SB as a gene transfer vector targeting therapeutically important organs in an *in vivo* gene therapy protocols. The results will lay the groundwork for optimization of this vector system and its future application to human gene therapy.

CRITIQUE 1:

SIGNIFICANCE: One of the most difficult problems in gene therapy today is the ability to stably express genes for long periods of time and not disrupt other aspects of cells biology. The mainstay of gene therapy continues to be viral vectors; however, these are fraught with problems including infection rates, oncogenicity, suppression, and limitations on genome size. All of these issues can be addressed by transposons to some extent. Thus, the development of novel strategies such as this may provide important methods of gene delivery, the foundation of gene therapy.

APPROACH: This application is divided into three aims. These aims have been modified from the previous application to be much more focused and address specific issues related to the use of SB transposon as a gene delivery system. Significantly, the investigators have made many attempts to address all the concerns of the reviewers and have directly spoken to the issues previously raised. They have also provided new preliminary and published data ensuring this project is moving forward.

Specific Aim 1 will investigate the safety of the SB transposon. Concern was raised that introduction of transposase might move dormant transposable elements and any movement could disrupt genes as the transposon leaves or as it inserts elsewhere in the genome. To this end the investigators have engineered cleaver insulator elements to make what they refer to as activation safe SB transposons. These will buffer the transposon promoter elements from inducing adjacent genes. They have devised a strategy to test this concern using the HSV-TK gene. Their strategy is relatively simple, and will certain address part of the question, although it still cannot address the induction of chromosomal genes that may be near the insertion site of a particular recombination. This is a difficult problem, and this reviewer cannot think of an experiment that would test this. More experience with the system will certainly provide answers over time.

In Specific Aim 2, the investigators will determine the efficiency of SB to transfer and express genes in the liver of mice. They have selected two mouse models to work with, the type VII mucopolysaccharidosis mutant mouse *Gus* (β-glucuronidase) and the ornithine transcarbamylase mutant mouse *Oct*. Both are well-characterized systems and are excellent animal models. It is not entirely justified why they are using two mutants to start when one would seem like plenty of work. Focusing on the *Gus* mutant probably would be sufficient to characterize how well SB will work to correct the enzyme deficiency. Both *trans*- and *cis*- organized systems of transposon and transposase delivery will be investigated. The investigators outline the use in detail of only one delivery system. Given that this is such an important facet of the problem facing gene therapy and the investigators are not sure this method would work in larger animals and humans, it would seem that they would want to investigate other delivery methods.

The investigators have outlined a strategy to detect the β-glucuronidase activity or the ornithine transcarbamylase activity, both approaches seeming reasonable. While nice plans are provided for analysis, it is unclear how many animals will be used in each experiment, and how comparisons will be made between groups. Some discussion of power calculations and interpretative plans would be helpful. This is particularly true for the behavioral testing where it is not even discussed what cognitive functions will be tested with the four paradigms and what is missed. Although the investigators are using the core to do this, but they nevertheless should show an understanding of what they are testing.

Specific Aim 3, like Aim 1, is relatively short but also addresses an important issue. Here the investigators will delineate how efficient fail-safe transposases are and how efficiently they will transpose SB and not affect other transposable elements. They provide a good strategy to evaluate the long-term expression from the fail-safe SB transposase and to monitor for additional transposing events. As with the other aims, very little is described on what they will do if they do not find there anticipated results, this is a flaw throughout this application. An additional concern is that it is not clear that this project uses the Cell Processing Core (Core D).

Finally, the investigators provide a nice response to the previous reviews on future directions into larger animals. They have set up collaborations with appropriate groups for doing this. The delivery system, as discussed above, could be a problem though.

INNOVATION: This is a highly innovative project. This is one of the first and continues to be one of the only laboratories working on transposable elements as a method for gene transfer. The investigators have pioneered many aspects of this field and continue to rigorously test the ability to use transposons as a method to constitutively express transgenes.

INVESTIGATOR: Dr. Perry Hackett and his colleagues on this project are ideally suited to carry out the proposed project. Dr. Hackett is Professor of Genetics and Cell Biology at University of Minnesota, St. Paul and Professor, Institute of Human Genetics, University of Minnesota, Minneapolis. In addition, he is Chairman and Chief Science Officer, Discovery Genomics, Inc. of Minneapolis. Dr. Hackett is a leader in the field of working with transposons and he has extensive experienced this area. He is uniquely qualified to carry out the proposed studies. In the previous application, the effort Dr. Hackett would be putting into this project was questions given his move to a private company. He has adequately addressed this issue. He maintains a faculty appointment at the University of Minnesota and spends at least 20% of his time at the University working on this project. The concern for his effort was amplified by a relative lack of productivity prior to the previous application. This too has been addressed by the publication of multiple papers on the SB transposon system over the past several years.

ENVIRONMENT: As stated above, this is an almost unique environment in which to carry out the proposed project. The space and resources are more than adequate and there is integration with other projects enhancing this project and the program.

CRITIQUE 2:

SIGNIFICANCE: This revised application proposes a series of experiments to demonstrate the possible utility of the Sleeping Beauty (SB) transposon as a means of delivering DNA for gene therapy. These studies could identify a new and efficient means of delivering genes for therapy that would minimize risk and maximize longevity of expression, which are highly significant.

APPROACH: As noted in the prior review, excellent progress has been made in this project during the previous award period.

Specific Aim 1, added in response to concerns raised at the previous critique, proposes two approaches to assure safety of the vectors. The first uses insulator sequences to limit or block aberrant expression of genes adjacent to transposon integration from promoters contained in the transposon. The second contains a "death gene" (viral thymidine kinase) that will kill cells containing the transposon when they are exposed to nucleoside analogs like gancyclovir. The experiments to verify the efficacy of these approaches are well thought-out and are highly likely to show whether the approaches work or not.

Specific Aims 2 is to determine the efficiency of SB for gene transfer in cells and in mice. Initial experiments at the cell level served to optimize the vector and SB, and to study some methods for delivery of the transposon plus transposase at the cell surface. These will be extended one-step further to deliver the vector *in vivo*. Preliminary data showing the ability to obtain transfection and integration in liver indicate that the approaches proposed are likely to succeed, and these experiments should measure how well that occurs as well as examine long-term expression in biologically relevant systems.

Specific Aim 3 will evaluate fail-safe vectors in cells and *in vivo*. Methods optimized in Aim 2 will be used to deliver the new vectors built in Aim 1 and to assess their efficacy for minimizing unintended effects of the transposons. These will be carried out initially in cells and then in mice to correct a GUS

(β-glucuronidase) genetic deficit. As indicated from these experiments, the system will ultimately be applied to dogs since large animals pose different issues of delivery as well as requiring optimization for a different biological system.

INNOVATION: The proposed experimental approach is highly innovative.

INVESTIGATOR: Dr. Perry Hackett, the principal investigator, is extremely well positioned to carry out these experiments. Concerns from the previous review about commitment are addressed appropriately, and the publication record from this project serves as a concrete demonstration of the principal investigator's commitment.

ENVIRONMENT: The resources at University of Minnesota are highly conducive to the studies proposed.

RECOMMENDATION: Project IV is recommended for further consideration as requested with a priority score of 141.

CORE FACILITIES
CORE A ADMINISTRATIVE CORE
(Chester Whitley, Ph.D., M.D., pp. 600-605)

DESCRIPTION (provided by applicant): The Administrative Core for this program project is an administrative subunit of the Department of Pediatrics. The main objective of this core is to provide a support system that assures continued research, productivity, communication among project investigators, and proper oversight in the management of public funds. This unit must be responsive to the individual needs of each project investigator and will provide several services. The Administrative Core includes the principal investigator, an administrative assistant, as well as support for regular meetings, five-member External Advisory Committee and ad hoc advisors, as well as access to patients for shared research specimens and for future clinical trials.

CRITIQUE: The Administrative Core for this program project is to be directed by Dr. Whitley and is within the Department of Pediatrics at the University of Minnesota School of Medicine. The core supports one administrative assistant who oversees all organizational aspects of the program project, including support for all meetings, the External Advisory Committee, and ad hoc advisors. In addition, this core provides and coordinates patient access for shared research specimens and for future clinical trials. The core will also provide assistance with grant development, planning and grant management, and word processing. Weekly meetings are arranged for all researchers interested in gene therapy for metabolic diseases. The projects rotate responsibility for the scientific content of the meeting. The core will also arrange meeting with the Internal and External Advisory Committee members, either individually or as a group.

There is a well-defined organization structure for the program project and the core will support the entire program. All of the listed services are appropriate. The only exceptions are providing access to the patients and shared research specimens. There is no description on how this will occur, how information is stored, and how access will be protected. Also, planning for future clinical trials is an enormous task and cannot be run with the resources provided by this core.

RECOMMENDATION: The Administrative Core is recommended for further consideration as requested for the duration of the program project.

CORE B MICROCHEMICAL CORE
(Charlotte Brown, Ph.D., pp. 650-653)

DESCRIPTION (provided by applicant): The Microchemical Core is an integral component of this program. Continuation of this work will be made possible by the use of expertise, equipment, and supplies provided by this core. In addition for providing a center for intellectual interactions, experimental design, trouble-shooting, and other consultation, the Microchemical Core operates as a service facility that provides a number of biologic reagents, such as oligonucleotide primers, which are heavily used in the proposed research. In addition, the core accomplishes automated DNA sequencing of transcripts (RT-PCR) products, and cloned DNA (i.e., plasmid, retrovirus constructs and AAV vectors). The facility will also provide mutation analysis of experimental cell lines and for molecular diagnosis of patients entering into a clinical trial. The high-capacity sequencing capability will also be required to validate vector constructs and, in the Gene Scan mode, for quantitative analysis of gene insertion into target cells. In addition, the core provides a host of other instrumentation for analysis and synthesis (such as for peptide sequencing, peptide synthesis, etc.) that will be used on a less frequent basis.

CRITIQUE: The Microchemical Core is directed by Dr. Charlotte Brown, and basically functions as a genetics core. It provides synthesized oligonucleotides, DNA sequencing, and mutational analysis. The core has high through put capacity for these services. In addition, the core also can perform protein sequencing and synthesis.

The core performs important services for each of the three projects. It is particularly heavily utilized in oligonucleotide synthesis and sequencing. The core is very capable to perform the proposed task, despite the fact that there is very little description of how this core will serve each of the projects.

RECOMMENDATION: The Microchemical Core is recommended for further consideration as requested with a priority score of 131.

CORE C RESEARCH ANIMAL CARE AND MEDICINE CORE
(Cynthia Gillett, D.V.M., pp. 700-703)

DESCRIPTION (provided by applicant): The multiple specialized mouse models of genetic dysfunction used for each of these projects require expert animal care, housing, and veterinary preventive medicine and diagnostic support. Analysis of strain needs, procurement and breeding options, and intensive management and monitoring of in-house breeding production colonies are necessary for the smooth functioning of the specific aims of these projects. The Research Animal Care and Medicine Core will provide veterinary oversight and individualized care and programming for the animal models integral to each project. This core will interact closely with the Quantitative PCR Core to provide the most appropriate samples (tissue or blood) for prompt genotyping and individual animal identification. In addition, the core will work closely with the Neurological Services Core to provide animals (homozygous, heterozygous, or wild-type) at the correct ages and times for comprehensive neurophenotyping.

CRITIQUE: The purpose of the Research Animal Care and Medicine Core is to breed and genotype the mouse lines used in the program project. The centralized care of these mice seems to be an efficient way to accomplish the important task of properly care and maintain the rodent lines required for the work in this program.

Cynthia Gillett, D.V.M., is Director of Research Animal Resources at University of Minnesota, Minneapolis. Dr. Gillett is board certified by the American College of Laboratory Animal Medicine and she has the expertise to carefully direct the breeding and care of the mouse strains.

Overall, this is an important core that should facilitate the care and maintenance of the mouse strains required for the work proposed in the program project.

RECOMMENDATION: The Research Animal Care and Medicine Core is recommended for further consideration as requested with a priority score of 140.

CORE D CELL PROCESSING CORE
(Jeffrey Miller, M.D., pp. 750-758)

DESCRIPTION (provided by applicant): The purpose of the Cell Processing Core is to obtain and provide human blood cells for investigations being carried out as part of the program project. Cells that will be provided through this core include peripheral blood stem cells, mononuclear cells, and umbilical cord blood cells. These cells will be obtained on a large scale from either normal donors or patients, and provided in the composition obtained from the donor or processed in the laboratory to isolate sub populations such as lymphocytes or $CD34^+$ cells. The core will also provide *in vitro* cell culture or clonogenic assay services for the measurement and evaluation of hematopoietic progenitor cells that have undergone gene transfer manipulations as specified in this program project. As projects move toward clinical application, the core will provide these same services for clinical trials at the GMP facility on the St. Paul Campus, the Molecular and Cellular Therapeutics Facility.

CRITIQUE: The Cell Processing Core appears to have state-of-the-art instruments for obtaining hematopoietic cells necessary for this program. The services provided include: G-CSF mobilization and collection of peripheral blood stem cells; isolation of $CD34^+$ cells (with a new high-end instrument, the Isolex 300i, that can do so with >90% efficiency); isolation of pluripotent hematopoietic stem cells; flow cytometry; large-scale transduction (principally evaluating conditions needed for viral vector efficiency, such as quantities and types of cytokines, vector presentation, transduction conditions, length of time in culture to determine optimal methods), and hematopoietic assays to assess transduced progenitor populations. The rigor with which hematopoietic progenitor types are defined is admirable. The core also has the ability to plate single cells robotically and follow their differentiation fate.

It is clear that this core is essential for Project I, the lentiviral *ex vivo* manipulation of human hematopoietic stem cells for therapy of MPS I. These blood cells – including those obtained from patients with MPS I – will be essential for testing the various parameters for optimal lentiviral infection and for ultimate transplantation into the *NOD/SCID* mice. The necessity of this core for the other two projects remains a bit obscure. This may be due to the fact that the details of the core's "Large Scale Transduction Services" remain vaguely defined. Project II, using AAV to correct Purkinje cells in SCA1 mice, is said to use the flow cytometric services offered by the core and one could envision attempting to do AAV titering on blood-derived cells (though that is not described in Project II). However, there appears to be no obvious relevance of this core for Project IV (the transposon project).

Overall, the quality of the work and leadership of this core is first-rate. The core is clearly necessary for Project I. Its importance for the other two projects remains unclear and should be addressed administratively.

RECOMMENDATION: The Cell Processing Core is recommended for further consideration as requested with a priority score of 151.

ADMINISTRATIVE NOTE: It is not apparent that this core is supporting all three projects as proposed in the application. If that is the case, then perhaps its service should be combined with another core facility.

CORE E REAL TIME QUANTITATIVE PCR CORE

DESCRIPTION (provided by applicant): The purpose of the Real-Time Quantitative PCR Core is to assist investigators in evaluating gene transfer efficiency and transgene expression in target cells for projects in the program. As initially demonstrated by a gene therapy clinical trial ("Lymphocyte gene therapy for Hunter syndrome"), this technique fills a very significant scientific need, to accurately quantitate the presence of a gene, and to measure its expression by reverse transcriptase-PCR (RT-PCR) with a high level of sensitivity and reproducibility. This core will assist investigators in designing assay systems (paired oligonucleotide primers and an internal TaqMan probe, Black Hole Quencher probe, or SYBR Green), as well as accomplish the actual assays of research specimens. The availability of a core facility accomplishing assays for common biologic systems (e.g., gene and gene products for OTC, MPS I, and MPS VII mouse models) eliminates redundancy and provides a greater level of quality assurance. The new, quantitative technology also provides sensitive methods for "safety assays" (e.g., for lentiviral vector RCR) that are much faster, reliable, and inexpensive in comparison to existing culture approaches. The technique also provides a method of quantitating gene products (RT-PCR) for which there are no existing assays, or for DNA sequences that do not yield a translational or transcriptional endproduct (as might be needed for transposon vectors).

CRITIQUE: Dr. Dao Pan will direct the Real-Time Quantitative PCR Facility. This core will provide RT-PCR for evaluating gene transfer efficiency and transgene expression in target cells. It will also provide pre-experiment consultation on the most appropriate methods in addition to methods of analysis. The core is divided into three aspects, design-related services, operation related services, and post-assay data analysis services. Each is well described. The design related services will participate in primer set selection and selecting appropriate controls and methods for interpretation. The operations related services will actually perform the QPCR and associated trouble shooting. The post-assay data analysis services will validate experiments using controls and gene specific standards, establish standard amplification curves and finally data archiving.

This core is very well organized and it has excellent abilities and track record. The facility is state-of-the-art. The core provides numerous example of how it has been functioning and indicates it has carefully carried out excellent and high quality QPCR with carefully planned controls. Unfortunately, there is some error in the write-up, including descriptions of servicing four projects instead of three, and miss naming the director. Utilization will be by all three of the project. Potential overlap between this core and Core B suggests perhaps these two cores can be combined.

RECOMMENDATION: The Real Time Quantitative PCR Core is recommended for further consideration as requested with a priority score of 131.

CORE F VIRAL VECTOR CORE
(Scott McIvor, Ph.D. & Nikunj Somia, Ph.D., pp. 850-862)

DESCRIPTION (provided by applicant): The purpose of the Viral Vector Core is to provide packaged virus vectors for investigations being carried out as a part of the program project. Recombinant DNA manipulations will be carried out in the laboratories of the individual investigators to generate specific vector constructs. These vector constructs will then be packaged in this core, relying on extensive experience of the vector core laboratory in the procedures carried out for these preparations. Vector types to be prepared for this program includes recombinant lentivirus vectors, adeno-associated virus vectors, and adenovirus vectors for Projects I, II, and IV, respectively. The core will also provide guidance on vector construct design and analysis for vector-mediated gene transfer into target cell populations.

CRITIQUE: The Viral Vector Core will provide AAV, lentiviral, and adenoviral vectors as well as vector consulting services for the program. The prior review had a few comments that have been addressed in this revised application

This is an unusually diverse vector core providing three distinct vector types that require very different procedures. Dr. Scott McIvor, a well-qualified investigator with extensive expertise in retroviral vectors, will be the director of this core. He has demonstrated expertise in the production of AAV vectors and adenoviral vectors in the application. The core co-director, Dr. Nikunj Somia, is an expert in lentiviral vectors. Together, these investigators will be able to provide the vectors for the Projects I, II, and IV. Ample discussion is given illustrated the core's ability to both produce and quality control for the vectors. Critical in all gene transfer vector preparations are the abilities to access for helper virus contamination. The procedures described seem adequate.

Environment for the core consists of 2,000 square feet of laboratory space with tissue culture and general laboratory equipment. The facilities described as well as the currant Good Manufacturing Practices viral vector core (directed by Dr. McIvor) are adequate to provide the services described.

RECOMMENDATION: The Viral Vector Core is recommended for further consideration as requested with a priority score of 149.

CORE G NEUROLOGICAL SERVICES CORE
(Walter Low, Ph.D., pp. 900-911)

DESCRIPTION (provided by applicant): The Neurological Services Core for this program project will provide support for the surgical delivery of therapeutic gene constructs into the brain of MPS I, MPS VII, and SCA1 mice that will be used in Project I (MPS I), Project II (SCA1), and Project IV (MPS VII). In addition, this core will be involved in the neurologic assessment of cognitive and motor functions in these animals, as well as the neurohistological assessments after the targeted surgical delivery of the therapeutic gene constructs.

CRITIQUE: This is a clearly necessary core for all the projects, and the quality of the service and leadership of the facility is excellent. Dr. Walter Low, the core director, is Professor of Neurosurgery and Physiology and Program in Neuroscience and a recognized expert in all of the techniques described.

The behavioral assessments performed by the core are all routinely used – and expected – in this field. Many of measures the motor function are particularly pertinent to assessing cerebellar function (Project II). The assessments include rotarod testing, gait width testing, and the elevated bridge test. Cognitive function measures (active avoidance test, open field hole board test, spatial reference memory, spatial navigation, and spatial working memory) are pertinent to assessing function of the hippocampus, a region affected in MPS I (Project I) and MPS VII (Project IV). All the projects will require histological processing and microscopic examination, including confocal microscopy. Most of the projects will require surgical implantation of therapeutic substances; in particular, Project II will require stereotactic implantation of AAV vectors into the cerebellum.

RECOMMENDATION: The Neurological Services Core is recommended for further consideration as requested with a priority score of 143.

OVERALL CRITIQUE:
PROGRAM AS AN INTEGRATED EFFORT: This revised program project brings together a distinct group of focused investigators who approach the problems of mental retardation with the novel therapeutic approach of gene therapy. The common goal of understanding basic science and then translating that to the clinic is the major strength of this application. The basic molecular genetic aspects in each of the three projects might eventually lead to more therapeutically oriented techniques

applicable not only for mental retardation disorders but multiple diseases with known genetic etiology. This revised application has done an excellent job of addressing concerns raised at the prior review.

The strength of the program lies precisely in the diversity of approaches and the leadership of each project with the overall program leadership under Dr. Chester Whitely. The focus of this program on trying to correct genetic defects associated with mental retardation is clearly one of its strengths. Also, using novel molecular techniques that can be used clinically has far reaching implications. The three projects cover almost the entire gamut of gene therapy, from *ex vivo* to *in vivo*, transduced cell-mediated, metabolic correction based on the bystander effect, to the use of advanced lentivirus-based vectors and of AAV-based vectors of different serotypes, to *trans*-splicing for correction of *trans*-dominant gene defects, to a non-viral transposon-based system to achieve transgene integration. While it cannot be predicted which, if any, of the different methodologies under study may be successful, and to which specific condition it may be applicable; however, by exploring a wide range of approaches in this type of program, the probability of finding one that works is certainly increased.

In addition to the unifying common theme of treating mental retardation disorders with gene therapy, the well-functioning cores shared by all projects, as well as the large number of regular, scheduled meetings for all investigators to discuss progress and plans provide additional means of integration among projects. Finally, the apparent outstanding leadership of the principal investigator in putting together this revised application should be noted. There is great confidence that the program project will continue its success in this difficult scientific area.

OVERALL MERIT OF THE PROGRAM: Therapy of genetic diseases with prominent neurological involvement is an extremely worthwhile endeavor, but it is fraught with numerous difficulties and limited clinical successes. This program attacks many of the problems by an array of diverse approaches, encompassing cell-mediated metabolic correction, development and testing of advanced viral vectors for transgene delivery and integration, and less established, but promising, new methods of non-viral transgene integration, all appropriately to be tested in adequate animal models. The principal investigator's commitment to translational research for therapeutic intervention in patients and his experience in clinical trails give reasonable assurance that positive results obtained in the more basic research projects, after critical evaluation in experimental animals, will eventually find their way to practical human application. Overall, each individual project in this revised application addressed the conceptual or methodological weaknesses pointed out in the prior review. Finally, the program appears to be reasonably focused and well integrated. Any perceived weakness has to do with the overall methodology of gene therapies that is simply adequate delivery issue to the CNS. Animal models may address this important point prior to human clinical trials. The investigators adequately understand this and make all attempts to remain focused and diligent in the field. Any limited success in the planned program should have a significant impact in the field of gene therapy and treatment of diseases with known genetic etiology.

INNOVATION: Each of the projects has innovative aspects, if not always in conceptual developments, at least in their application. Thus, Project I proposes to utilize lentiviral vectors in a new animal model of MPS I, and to explore the effectiveness of *in vivo* transfection of HSC. Project II proposes genetic correction of a *trans*-dominant mutation. Finally, Project IV proposes to explore a novel method of transgene integration based on a transposon system.

INVESTIGATORS: The program director, the principal investigators in the individual projects, the core directors, and their collaborators are all well qualified to carry out the proposed studies. Any question raised about the commitment of Dr. Hackett to this program was adequately addressed in the revised application.

ENVIRONMENT: In the previous two reviews, which included a site visit, the resources were deemed excellent. The prior perception of apparent lack of strong institutional support was addressed as demonstrated by the commitment of facilities. From the description of the program itself and of the

various entities with which the program interacts (such as the BMT Program and the Molecular Therapeutics Program), the environment can be considered conducive to the proposed research.

CLINICAL RESEARCH: In Projects I, the proposed experiments require tissues donated from normal adults or subjects with mild MPS I. Studies are then conducted *in vitro* that cannot be traced to a living individual in accordance with the new Health Insurance Portability and Accountability Act guidelines.

SCIENTIFIC REVIEW ADMINISTRATOR'S NOTES: It is not apparent that the Cell Processing Core (Core D) is supporting all three projects as proposed in the revised application. If that is the case, then perhaps its service should be combined with another core facility.

Reviewers also note the similar services to be provided by the Microchemical Core (Core B) and the Real Time Quantitative PCR Core (Core E). Perhaps these two facilities can be combined into a more cost-effective unit.

THE FOLLOWING RESUME SECTIONS WERE PREPARED BY THE SCIENTIFIC REVIEW ADMINISTRATOR TO SUMMARIZE THE OUTCOME OF DISCUSSIONS OF THE REVIEW COMMITTEE ON THE FOLLOWING ISSUES:

PROTECTION OF HUMAN SUBJECTS (Resume): ACCEPTABLE

VERTEBRATE ANIMAL (Resume): ACCEPTABLE

COMMITTEE BUDGET RECOMMENDATIONS: The budget was recommended as requested.

Ad hoc or special section application percentiled against "Total CSR" base.

NOTICE: The NIH has modified its policy regarding the receipt of amended applications. Detailed information can be found by accessing the following URL address: http://grants.nih.gov/grants/policy/amendedapps.htm

NIH announced implementation of Modular Research Grants in the December 18, 1998 issue of the NIH Guide to Grants and Contracts. The main feature of this concept is that grant applications (R01, R03, R21, R15) will request direct costs in $25,000 modules, without budget detail for individual categories. Further information can be obtained from the Modular Grants Web site at http://grants.nih.gov/grants/funding/modular/modular.htm

MEETING ROSTER

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NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT
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* Temporary Member. For grant applications, temporary members may participate in the entire meeting or may review only selected applications as needed.

Consultants are required to absent themselves from the room during the review of any application if their presence would constitute or appear to constitute a conflict of interest.